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Extreme mitochondrial population subdivision in southern Appalachian paleoendemic spiders (Araneae: Hypochilidae: *Hypochilus*), with implications for species delimitation

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Abstract. A prior study of molecular phylogenetic relationships in southern Appalachian *Hypochilus* taxa revealed unusually high intraspecific mitochondrial sequence divergences, but was limited by small intraspecific sample sizes. A subsequent in-depth population genetic study focused on a single species (*H. thorelli* Marx 1888), revealing genetic patterns consistent with extremely limited female-based gene flow among rock-outcrop limited populations. Here we extend the study of mitochondrial population genetic structuring to four remaining Appalachian *Hypochilus* species. Genetic inferences are based on a sample of COI mitochondrial sequences generated for over 250 specimens from 85 sampled locations. This geographic sample comprehensively covers the geographic distributions of all described taxa. Phylogenetic, network-based, and genealogical sorting index analyses reveal ubiquitous genetic structuring in all *Hypochilus* taxa. A majority of sampled locations possess limited genetic variation, with site-specific haplotypes forming genealogically exclusive "microclades", consistent with limited female-based gene flow at the spatial scales sampled. At deeper phylogenetic levels, four of five described species are recovered as monophyletic on mitochondrial gene trees. *Hypochilus pococki* Platnick 1987 is recovered as paraphyletic, and is fragmented into five genetically divergent, allopatric phylogroups. These phylogroups, and multiple clades within one of the *H. pococki* phylogroups, are also recovered as distinct clusters in a generalized mixed Yule-coalescent (GMYC) analysis, suggesting the possibility of multiple cryptic species in the Appalachian fauna. However, a qualitative survey of male palpal variation fails to reveal morphological differences that distinguish these highly divergent genetic lineages. We suggest that a nuclear gene tree perspective is ultimately needed to resolve this contrast.

Keywords: Cryptic species, genealogical sorting index, GMYC model, population subdivision

The uplands that comprise the several physiographic provinces of the southern Appalachian Mountains are ancient. Uplifted during the Paleozoic, highlands of this erosional landscape have been available for biotic evolution throughout the Cenozoic. Some authors contend that certain elements of the modern fauna in fact have histories that reach to the Mesozoic or Paleozoic eras (Dillon & Robinson 2009). More recently, the region has been impacted by climatic variation, and it is hypothesized that the southern Appalachians served as refugia for many taxa during the Pleistocene glaciations (e.g., Church et al. 2003; Crespi et al. 2003; Walker et al. 2009). This combination of climatic variability and long-term availability, in concert with high topographic complexity, has fostered remarkable in situ evolutionary diversification. The southern Appalachians today represent one of the most biodiverse regions in the northern hemisphere (Stephenson et al. 1993; Stein et al. 2000), comprising a hotspot for short-range endemic aquatic and upland taxa. In upland animal taxa, endemic radiations are seen, for example, in millipedes (Marek & Bond 2006, 2009; Marek 2010), spiders (Hedin 1997; Hendrixson & Bond 2005), harvestmen (Thomas & Hedin 2008; Hedin & Thomas 2010), salamanders (Crespi et al. 2003; Weisrock et al. 2006; Kozak & Wiens 2010), and many other cryophilic groups.

The spider genus *Hypochilus* is one of the most distinctive spider groups in North America, representing the most early-diverging lineage (Family Hypochilidae) of "true" spiders (Platnick 1977; Forster et al. 1987; Platnick et al. 1991). *Hypochilus* shows a fragmented continental distribution, with species found in the southern Rocky Mountains, montane areas of California and the southern Appalachian Mountains (Catley 1994; Hedin 2001). The monophyletic southern Appalachian fauna (Catley 1994; Hedin 2001) includes five described species (*H. gertschi* Hoffman 1963, *H. thorelli* Marx 1888, *H. pococki* Platnick 1987, *H. sheari* Platnick 1987 and *H. coylei* Platnick 1987) distributed in strict allopatry across six states, from northern Alabama and Georgia to West Virginia (Fig. 1). Several lines of evidence suggest that Appalachian *Hypochilus* are both ecologically and morphologically conservative. All eastern species prefer relatively mesic habitats, and are almost always found on rock outcrops, where they build distinctive "lampshade" webs. Different species are sometimes found in adjacent locations on the same geologic outcrop (e.g., *H. thorelli* and *H. pococki* on Cumberland Mountain in southwest Virginia; Fig. 1), but multiple species have never been collected at the same site, indicating that ecological similarity (niche conservatism) may preclude syntopy. Appalachian *Hypochilus* are extremely similar in somatic morphology, distinguished only by subtle differences in male and female genital morphology (Forster et al. 1987; Huff & Coyle 1992; Catley 1994).

Prior research clearly shows that these spiders are also dispersal limited. Based on sparse phylogeographic sampling, Hedin (2001) revealed deep mitochondrial divergences within Appalachian species. Hedin and Wood (2002) conducted a more thorough mitochondrial study of *H. thorelli*, revealing high intraspecific mitochondrial divergences and fractal genetic structuring. Mitochondrial sequences from all sampled locations formed genealogically exclusive clades, regardless of the geographic proximity of sample sites. Although no quantitative morphological assessment was conducted, the authors noted no differences in genitalic morphology between

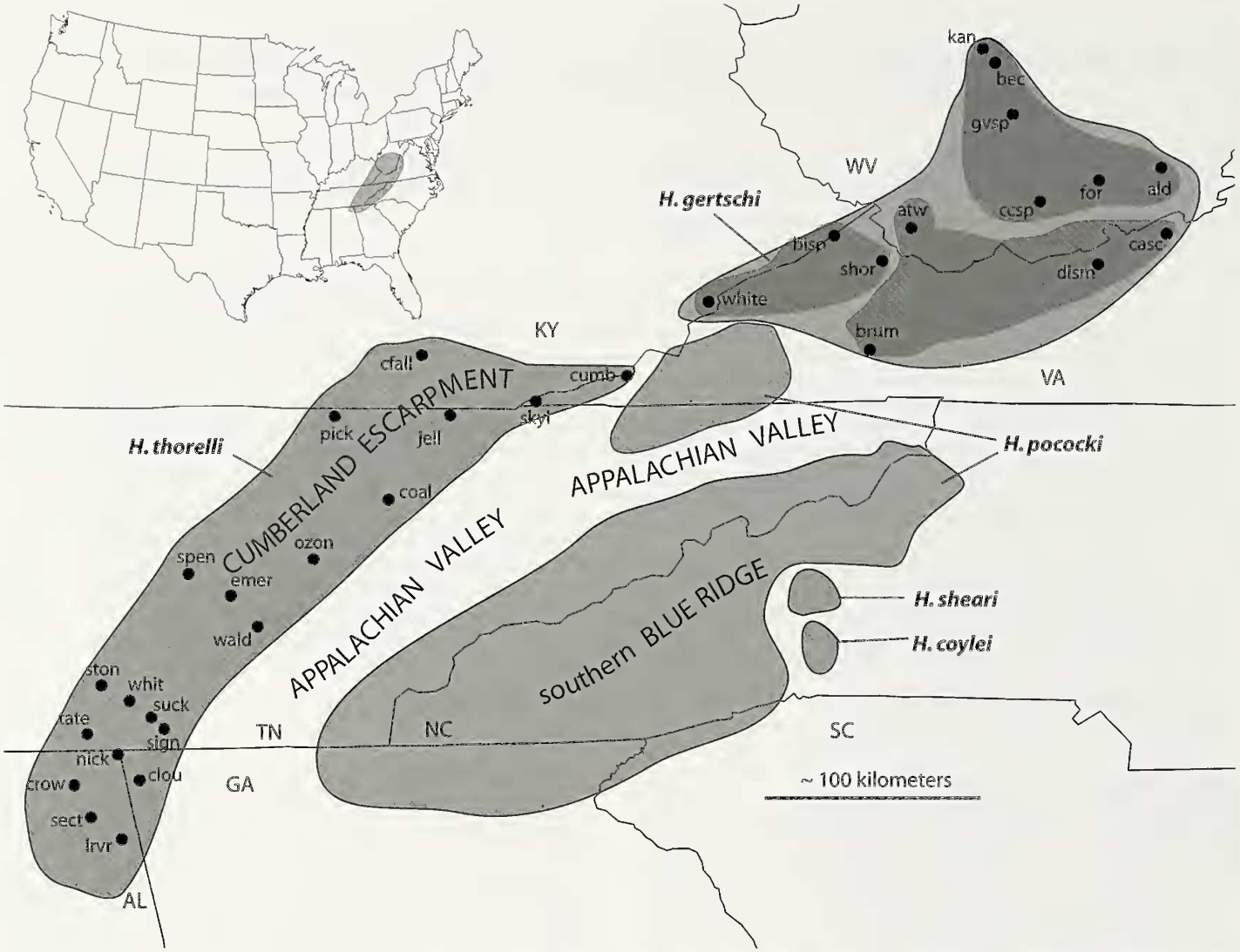

Figure 1.—Map of southern Appalachian region showing physiographic provinces and general distribution of eastern *Hypochilus*, with sampled sites for *H. thorelli* and *H. gertschi* (site acronyms found in Table 1). Geographic subclades consistently recovered in alternative RAxML analyses indicated by darker shading for *H. gertschi*.

populations, providing further evidence for morphological conservatism (i.e., morphological cohesion despite limited female-based gene flow).

Here we extend our studies of mitochondrial population structure and phylogeography to all described species of Appalachian *Hypochilus*, addressing two primary questions regarding genetic population structure and divergence. First, using a large genetic sample we investigate whether other Appalachian *Hypochilus* species show nearly complete mitochondrial population subdivision, as observed in *H. thorelli*. Appalachian taxa share many biological similarities, but also differ in important ways that might impact patterns of genetic structuring (e.g., relative range size, latitudinal position, etc., Fig. 1). Second, we use mitochondrial sequence data to detect possible cryptic species lineages within the Appalachian *Hypochilus* fauna. To address this second question we use standard gene tree patterns (e.g., do nominate taxa form genetic clades?), combined with methods of species delimitation derived from coalescent theory. For "candidate" cryptic lineages we also qualitatively assess geographic variation in male palpal morphology.

METHODS

Sampling.—Specimens representing the five Appalachian species were collected as follows: *H. pococki* (159 individuals from 56 sites), *H. gertschi* (61 individuals/13 sites), *H. sheari* (21 individuals/8 sites), *H. coylei* (18 individuals/6 sites) and *H. thorelli* (2 individuals/2 sites) (Figs. 1, 2; Table 1). DNA sequences gathered from these specimens were combined with previously collected data (Hedin 2001; Hedin & Wood 2002): *H. pococki* (4 individuals/4 sites), *H. gertschi* (2 individuals/2 sites), *H. sheari* (2 individuals/2 sites) and *H. thorelli* (18 individuals/18 sites). Collecting locations were approximately uniformly spread over the known range of each species, with a majority of neighboring sites separated by 20–40 km. Species with smaller distributions were sampled at a finer geographic scale (e.g., *H. coylei* sites separated by ~10 km). At any given site, specimen collection was dispersed (e.g., different regions

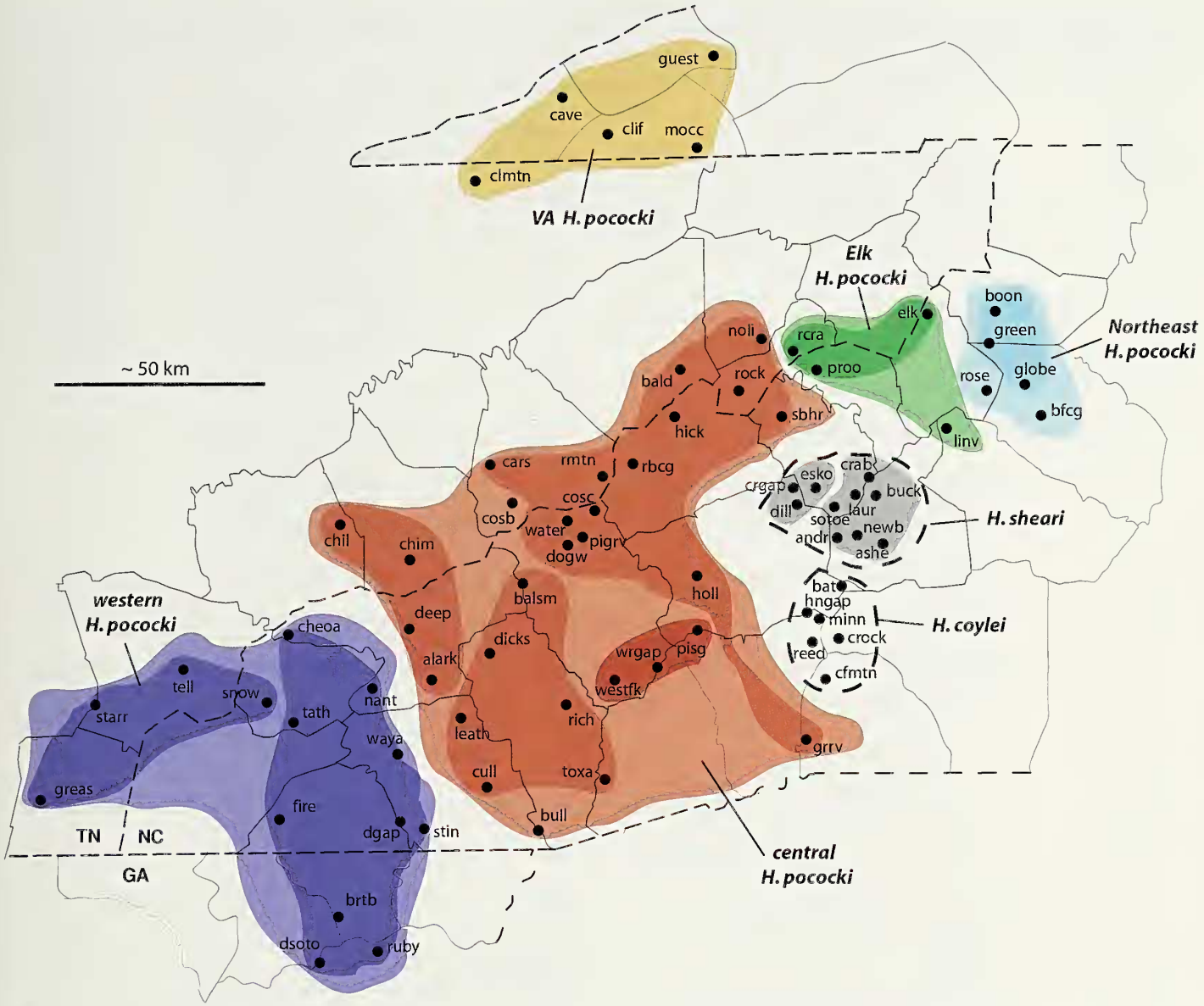

Figure 2.—Map of sampled sites for *H. pococki, H. sheari,* and *H. coylei,* mostly in the southern Blue Ridge Province. Site acronyms are found in Table 1. Primary geographic clades of *H. pococki,* as recovered in phylogenetic analyses, are individually colored. Geographic subclades consistently recovered in alternative RAxML analyses indicated by darker shading.

of a rock face) as we attempted to reduce the probability of collecting related individuals. Specimens intended for molecular work were preserved in 100% EtOH in the field. Because there are no known instances of species sympatry in eastern *Hypochilus* (Catley 1994), we sometimes used immature specimens for genetic analysis; immature specimens were always associated with a sample of adult voucher specimens (preserved in 80% EtOH) from the same location. Adult specimens were identified to species using diagnostic characters following Forster et al. (1987), Huff and Coyle (1992) and Catley (1994). Voucher specimens for all species, and all major phylogeographic clades within species (see Results), have been deposited at the California Academy of Sciences.

Molecular techniques.—Genomic DNA was extracted from leg tissues using the DNeasy Kit (Qiagen). Genomics were used as templates in PCR (Polymerase Chain Reaction) experiments, targeting an approximately 900 bp fragment of the mitochondrial cytochrome oxidase I (COI) gene region. This is the same gene region used by Hedin and Wood (2002), allowing a direct comparison between datasets. This gene region also overlaps the "DNA barcoding" locus used for spiders by Robinson et al. (2009). PCR experiments included an initial 94°C denaturation followed by 30 cycles of 45 s at 94°C, 45 s at 45°C, 90 s at 72°C, with a final 10-min extension at 72°C. Primers utilized are shown in Table 2. All PCR experiments included *Ex Taq* (Takara Bio, Inc.) with manufacturer-provided dNTP mixture and *Ex Taq* buffer (Mg^{2+}). PCR amplification products were purified via Polyethylene Glycol (PEG) precipitation, or by using an *IsoPure* PCR Purification and Gel Extraction Kit (Denville Scientific, Inc.). PCR products were sequenced using Big Dye Version 3 dye chemistry (ABI) on ABI 377 and Prism 3100

Table 1.—Taxon identity, locality information, site acronym, genetic diversity (observed maximum number of nucleotide site differences per geographic location), specimen number(s), and GenBank accession numbers. Bolded voucher number sequences submitted to GenBank. Adult male spiders were examined from those sites with site acronyms highlighted by an asterisk.

Species	Locality	Acronym	Max diff.	Hedin Lab #	GenBank acces. no.
H. pococki "central"	TN: Cocke Co., SE Round Mountain, W Rattlesnake Gap, 35.8471, −82.9443	rmtn	1	**H350=H351**, H352	JQ974835
"	NC: Madison Co., Hwy 209, W Rocky Bluff CG @ Long Mtn Branch, 35.8599, −82.8502	rbcg	7	H356, H359, **H360**	JQ974836
"	NC: Madison Co., East Prong Hickory Fork Creek, 35.9900, −82.52448	hick	12	H372, **H373=H375**	JQ974837
"	TN: Greene Co., Bald Mtns, E Greystone Mtn, Round Knob Road, 36.0799, −82.6859	bald	2	**H678=H679**, H680	JQ974838
"	NC: Polk Co., Green River Cove Road, 35.2539, −82.3301	grvr	0	**H489=H491=H492**	JQ974839
"	TN: Unicoi Co., E Rocky Fork, just up Edwards Branch road, 36.0662, −82.5245	rock	7	H377, H378, **H379**	JQ974840
"	TN: Washington Co., Little German Rd, along Nolichucky River, 36.1680, −82.4675	noli	1	**H694=H695**, H696	JQ974841
"	NC: Yancey Co., 19W, along Cane River, vic. Snakebite Holler Road., 35.9496, −82.3837	*sbhr	1	H674, **H673=H675**	JQ974842
"	NC: Haywood Co., Cold Springs Creek, 35.7594, −82.9953	cosc	1	H344, **H345=H346**	JQ974843
"	NC: Haywood Co., Dogwood Flats Creek, W Longarm Mtn, 35.7201, −83.0731	*dogw	4	H333, **H332=H334**	JQ974844
"	NC: Haywood Co., S. Waterville, Flat Branch Crk (of Mt Sterling Crk), 35.7407, −83.0741	water	6	H328, **H326=H327=H329**	JQ974845
"	NC: Haywood Co., FR 288 above Pigeon River, 35.7260, −83.0265	pigrv	2	**H338=H339**, H340	JQ974846
"	TN: Cocke Co., Carson Springs Road, 35.9411, −83.2567	cars	1	H689, **H690=H691**	JQ974847
"	NC: Jackson Co., Dicks Creek, near Dicks Creek Church, 35.4056, −83.2586	dicks	1	**H597=H598**, H599	JQ974848
"	NC: Swain Co., GSMNP, road to Balsam Mtn, N Black Camp Gap, 35.5437, −83.1679	balsm	7	H629, H630, **H631**	JQ974849
"	NC: Transylvania Co., near Toxaway Falls, 35.1247, −82.9297	*toxa	0	**H655=H656=H657**	JQ974850
"	NC: Jackson Co., Rich Mtn, SE Sugar Creek Gap, 35.2907, −83.0040	rich	3	H608, H609, **H610**	JQ974851
"	NC: Macon Co., NE Leatherman, 35.2965, −83.3666	leath	1	H664, **H666**	JQ974852
"	TN: Sevier Co., GSMNP, Chimneys @ Hwy 441, 35.6417, −83.4818	chim	–	–	AF303511
"	NC: Swain Co., GSMNP, Deep Creek, 35.4644, −83.4344	deep	0	**H623=H624=H625**	JQ974853
"	TN: Blount Co., Chilhowee Mountain, near Walland, 35.7331, −83.8165	*chil	1	H706, **H705=H707**	JQ974854
"	NC: Swain Co., Alarka Road, N Deep Gap church, 35.3482, −83.4064	alark	1	H594, **H593=H595**	JQ974855
"	NC: Transylvania Co., Hwy 276, 2 mi. S BRP, S Wagon Road Gap, 35.3682, −82.7862	wrgap	5	H500, **H501**	JQ974856
"	NC: Buncombe Co., NE Mt. Pisgah, head of McKinney Creek, 35.4448, −82.7225	pisg	0	**H639=H640**	JQ974857
"	NC: Haywood Co., Hwy 215, near head West Fork Pigeon river, 35.3390, −82.9016	*westfk	1	H635, **H633=H636**	JQ974858
"	NC: Macon Co., Chattooga River @ Bullpen bridge, 35.0172, −83.1262	bull	1	H613, **H615=AF303512**	AF303512
"	TN: Cocke Co., GSMNP, trail from Cosby to Low Gap, 35.7374, −83.1813	cosb	18	H322, **H321=H323**	JQ974859
"	NC: Macon Co., Cullasaja River Gorge, 35.0803, −83.2578	cull	2	H603, H604, **H605**	JQ974860
"	NC: Buncombe Co., Holland Mtn., Dogwood Road, S of Newfound, 35.6008, −82.7259	holl	5	H770, H773	JQ974861

Table 1.—Continued.

Species	Locality	Acronym	Max diff.	Hedin Lab #	GenBank acces. no.
H. pococki "Virginia"	VA: Lee Co., Cave Spring Recreation Area, NE Penington Gap, 36.8033, −82.9210	cave	0	**H232=H233**	JQ974862
"	VA: Wise Co., above Guest River, 36.9009, −82.4147	*guest	1	H742, **H740=H743**	JQ974863
"	VA: Scott Co., Cliff Mtn, 36.7495, −82.7787	*clif	0	**H733=H734=H735**	JQ974864
"	TN: Hancock Co., Hwy 31 on Clinch Mtn, 36.413, −83.2237	*clmtn	7	**H754**, H756	JQ974865
"	VA: Scott Co., Hwy 23/58/421 @ Moccasin Gap, 36.6338, −82.5550	*mocc	–	**H763**	JQ974866
H. pococki "Northeast"	NC: Watauga Co., West of Boone @ Watauga Rvr Crossing, Hwy 194, 36.1943, −81.7451	boon	1	H411, **H410=H413**	JQ974867
"	NC: Watauga/Caldwell Co., Green Mtn., Hwy 221 @ Green Mtn. Creek, 36.1142, −81.7782	*green	0	**H426=H427=H428**	JQ974868
"	NC: Avery Co., Roseboro Road, past first crossing Rockhouse Crk, 36.0192, −81.7813	*rose	0	**H432=H433=H434**	JQ974869
"	NC: Caldwell Co., Boone Fork CG, S of Chestnut Mtn, 36.0071, −81.6166	*bfcg	1	**H416=H417, H418**	JQ974870
"	NC: Caldwell Co., Globe Mountain Road, near Globe Mtn gap, 36.029, −81.667	*globe	0	**H422=H424=H425**	JQ974871
H. pococki "western"	NC: Graham Co., along Snowbird Creek, near Wilson Cabin, 35.2733, −83.9051	snow	0	**H572=H573=H574**	JQ974872
"	NC: Graham Co., Snowbird Mtns, N Tatham Gap, head Long Creek, 35.2579, −83.8196	tath	1	**H577=H578, H579**	JQ974873
"	NC: Swain Co., GRSMNP, along Lake Cheoah, Hwy 28, 35.4644, −83.8866	*cheoa	0	**H582=H584=H585**	JQ974874
"	TN: Polk Co., Hwy 64, along Lake Ocoee, 0.25 mi. E Greasy Crk bridge, 35.1112, −84.5647	*greas	0	**H546=H547=H548**	JQ974875
"	NC: Macon Co., W Wayah Depot, 35.1594, −83.5271	*waya	14	H643, **H644=H645**	JQ974876
"	NC: Clay Co., Fires Creek, W Omphus Ridge, 35.1029, −83.8435	fire	2	**H526=H527**, H528	JQ974877
"	GA: Towns Co., road to Brasstown Bald, 34.8593, −83.8008	brtb	19	H531, H533, **H534**	JQ974878
"	GA: White Co., Anna Ruby Falls Rec Area, 34.7576, −83.7101	*ruby	0	H536=H539=H540	JQ974879
"	GA: Lumpkin Co., DeSoto Falls Rec Area, trail to Upper Falls, 34.7062, −83.9153	dsoto	16	H541, H542, **H543**	JQ974880
"	TN: McMinn Co., N end of Starr Mountain, 35.3420, −84.4076	starr	3	H551, H553, **H554**	JQ974881
"	TN: Monroe Co., Tellico River, near Bald River Falls, 35.3248, −84.1787	tell	10	H556, **H558=H559**	JQ974882
"	NC: Swain Co., Nantahala River Gorge, 0.2 mi NE Blowing Spring, 35.32347, −83.63085	nant	1	H505=H506=H507	AF303513
"	NC: Macon Co., 4.3 mi S Standing Indian CG, 35.0347, −83.5057	*stin	0	**H512=H513=H514**	JQ974883
"	NC: Macon Co., 0.2 mi. N Deep Gap, 35.0425, −83.5550	dgap	0	**H517=H518=H519**	JQ974884
H. pococki "Elk"	NC: Burke Co., Linville Gorge, opposite Bull branch, 35.9396, −81.9219	*linv	6	H438, H437=H440	AF303514
	NC: Mitchell Co., Pigeonroost Creek, N of Nolichucky River, 36.0983, −82.2831	*proo	0	**H383=H387=H388**	JQ974885
"	NC: Avery Co., Elk River Cave, ~ 1 mi S Elk River Falls, 36.1892, −81.9617	*elk	2	H401, H402, **H403**	JQ974886
"	TN: Unicoi Co., Rock Creek Rec Area, 36.1379, −82.3482	*rcra	3	**H711**, H713	JQ974887
H. sheari	NC: Buncombe Co., W Cane River Gap, Hwy 197, 35.8036, −82.3536	crgap	0	**H444=H447=H448**	JQ974888
"	NC: Buncombe Co., Walker branch of Dillingham Creek 35.7677, −82.3594	*dill	3	H449, H450, **H451**	JQ974889

Table 1.—Continued.

Species	Locality	Acronym	Max diff.	Hedin Lab #	GenBank acces. no.
"	NC: McDowell Co., Newberry Crk (above Horse Br), N of Old Fort, 35.6825, −82.2170	newb	0	**H362=H363**	JQ974890
"	NC: Yancey Co., S. Big Laurel Mtn., N off BRP, 35.7401, −82.1991	*laur	0	**H364=H366**	JQ974891
"	NC: Yancey Co., South Toe River, below Chestnut knob, 35.7265, −82.2452	sotoe	6	**H370**, H371	JQ974892
"	NC: McDowell Co., Hwy 80, along Buck Creek, 35.7606, −82.1572	buck	0	**H454=H456=H457**	JQ974893
"	NC: McDowell Co., Andrew's Geyser, S side of Mill Creek, 35.6507, −82.2433	*andr	0	**H460=H461=H462**	JQ974894
"	NC: Yancey Co., Cane River, N Eskota, 35.8014, −82.3124	*esko	0	**H669=H670=H671**	JQ974895
"	NC: Yancey Co., Crabtree Falls	*crab	–	–	AF303515
"	NC: McDowell Co., US 70, E of Asheville	ashe	–	–	AF303516
H. coylei	NC: Buncombe Co., NW Hickory Nut Gap, Hwy 74, 35.4898, −82.3627	*hngap	2	H469, **H467=H468=H470**	JQ974896
"	NC: Rutherford Co., Chimney Rock Park, 35.4307, −82.2482	crock	1	**H473=H475**, H476	JQ974897
"	NC: Henderson Co., below Minnihaha Falls, Hwy 9, 35.4603, −82.2880	*minn	3	**H478=H481**, H479, H480	JQ974898
"	NC: Henderson Co., Reedypatch Creek, Hwy 64, W Little Fork Mtn, 35.4355, −82.3024	*reed	1	H484, H487=H488	JQ974899
"	NC: Polk Co., Cliffield Mountain, 35.3468, −82.2705	*cfmtn	1	H652, **H651=H653**	JQ974900
"	NC: Buncombe Co., below Round Mtn, Bat Cave road, 35.5314, −82.2202	*bat	–	H648	JQ975901
H. gertschi	VA: Washington Co., Brumley Creek @ Brumley Gap, 36.7933, −82.0229	brum	2	H728, **H729=H730**	JQ974902
"	VA: Buchanan Co., ~ 2 mi. W entrance Breaks Interstate SP, Hwy 80, 37.3012, −82.2880	bisp	6	H190, H191, H192, H193, **H194**	JQ974903
"	KY: Letcher Co., S Whitesburg, Hwy 199 @ summit of Pine Mtn, 37.0750, −82.8100	*white	8	**H236=H237**, H238=H239, H240	JQ974904
"	VA: Giles Co., Cascades of Little Stony Creek, ~ 2.5 mi. E of trailhead, 37.3643, −80.5792	casc	1	H221=H222, **H220=H223=H224 (=AF303519)**	AF303519
"	VA: Giles Co., Dismal Falls, 37.1878, −80.9003	dism	0	**H210=H211=H212=H213=H214=H215**	JQ974905
"	VA: Buchanan Co., 6 mi. W Shortt Gap, Hwy 460, along Levisa Fork, 37.1887, −81.9523	*shor	1	**H200=H201=H203, H204**	JQ974906
"	WV: McDowell Co., Hwy 83 @ Atwell, 37.3468, −81.7635	*atw	0	**H180=H181=H182=H183=H184**	JQ974907
"	WV: Fayette Co., Hwy 60, 0.5 mi. SW Kanawha Falls 38.1430, −81.2125	*kan	21	**H170=H171=H173**, H172=H174	JQ974908
"	WV: Fayette Co., ~ 1 mi. N Beckwith, along Laurel Creek, 38.1062, −81.1493	bec	0	**H160=H161=H162=H163=H164**	JQ974909
"	WV: Raleigh Co., W Grandview SP, 1 mi. E jnt Hwys 41/61, 37.8465, −81.1223	gvsp	1	**H150=H151=H152**, H153	JQ974910
"	WV: Mercer Co., Camp Creek SP, vic. Campbell Falls trailhead, 37.5092, −81.1337	*ccsp	1	H140=H141=H142=H143	AF303518
"	WV: Summers Co., E Forest Hill, along Spruce Run, 37.5906, −80.7913	for	0	**H130=H131=H132=H133=H134**	JQ974911
"	WV: Greenbrier Co., Rt 63 along Greenbrier River, 2 mi. E Alderson, 37.7308, −80.5955	ald	0	**H120=H121=H122=H123=H124**	JQ974912
H. thorelli	TN: Marion Co., Tate Spring Cave, se of Monteagle, 35.1770, −85.8073	tate	–	**H683**	JQ974913
"	VA: Lee Co., Cumberland Mtn, Wagonroad Tunnel Trail, 36.7308, −83.2207	cumb	–	**H802**	JQ974914
"	VA: Lee Co., Cumberland Gap NP, vic. Skylight Cave, 36.6165, −83.6443	*skyl	–	–	AF303510
"	KT: Whitley Co., Hwy 90, ~ 2 mi. E Cumberland Falls SP, 36.8474, −84.3083	cfall	–	–	AY102038

Table 1.—Continued.

Species	Locality	Acronym	Max diff.	Hedin Lab #	GenBank acces. no.
"	TN: Campbell Co., E of Jellico, Hwy 25W, 36.5756, −84.0691	*jell	–	–	AY102039
"	TN: Morgan Co., NW Coalfield, Hwy 62, Little Brushy Mtn, 36.0513, −84.4389	coal	–	–	AY102042
"	TN: Pickett Co., Pickett SF, Hwy 154 @ Natural bridge, 36.5452, −84.7976	pick	–	–	AY102043
"	TN: Cumberland Co., Ozone Falls, Hwy 70, 35.8805, −84.8103	*ozon	–	–	AF303509
"	TN: Van Buren Co., 0.5 mi. E Spencer, Hwy 30, 35.7319, −85.4321	spen	–	–	AY102046
"	TN: Bledsoe Co., Hwy 30 @ Emery Mill, W Pikeville, 35.6517, −85.1827	emer	–	–	AY102049
"	TN: Rhea Co., near Walden Ridge, Hwy 30 ~ 4 mi. W Dayton, 35.5298, −85.0495	wald	–	–	AY102050
"	TN: Grundy Co., Savage Gulf NA, Stone Door, 35.4397, −85.6487	*ston	–	–	AY102051
"	TN: Marion Co., ~ 5 mi. NW Whitwell, Hwy 108, ~ 1 mi S Star Gap, 35.2398, −85.5123	whit	–	–	AY102054
"	TN: Marion Co., Hwy 27, along Suck Creek, 35.1456, −85.3898	suck	–	–	AY102056
"	TN: Hamilton Co., Signal Mountain, vic. Chattanooga, 35.1193, −85.3477	sign	–	–	AF303508
"	GA: Dade Co., Cloudland Canyon SP, NW side Daniel Creek, 34.8343, −85.4843	clou	–	–	AY102061
"	AL: Jackson Co., Nickajack Cove, Hwy 73, 34.9804, −85.6094	nick	–	–	AY102063
"	AL: Jackson Co., Crow Mtn, below Clemmons Pt, Co. Rd 33, 34.8169, −86.0258	*crow	–	–	AY102064
"	AL: Jackson Co., NE side of Section, Hwy 35, 34.5955, −85.9981	sect	–	–	AY102066
"	AL: DeKalb Co., Little River Canyon, 34.3642, −85.6599	lrvr	–	–	AY102067
H. bonnetti	CO: Fly Cave				AF303525
H. kastoni	CA: West Boulder Lake				AF303521
H. bernardino	CA: Camp Creek				AF303524

capillary machines. Sequence contigs were assembled and edited using Sequencher version 4.2.2, and manually aligned using MacClade version 4.06 (Maddison & Maddison 2003). Sequence alignment was trivial, as no indels were present. Published COI sequences of *H. bonnetti* Gertsch 1964 (AF303525) from Colorado, as well as *H. kastoni* Platnick 1987 (AF303521) and *H. bernardino* Catley 1994 (AF303524) from California were used to root phylogenetic trees (sequences from Hedin 2001).

Phylogenetic and network analysis.—Identical haplotypes, except those shared among collection sites (less than five total haplotypes), were merged in MacClade prior to phylogenetic analysis. Gene trees were estimated using maximum likelihood (ML); rapid ML searches were conducted using RAxML version 7.0.4 (Stamatakis et al. 2008), implemented through the CIPRES (Cyberinfrastructure for Phylogenetic Research) portal v1.13. Searches included 100 rapid bootstrap replicates with a subsequent thorough ML search, assuming a GTR + G model. To explore alternative partitioning strategies, three separate RAxML analyses were conducted [unpartitioned, 2 partitions (first plus second, third), 3 partitions (first, second, third positions)]. For a subset of closely-related sequences that showed patterns of haplotype sharing among collection sites (see Results), haplotype networks were constructed using the program TCS v. 1.21 (Clement et al. 2000).

Genealogical sorting index.—The genealogical sorting index (*gsi*) statistic (Cummings et al. 2008) was used to quantify the degree of genealogical clustering of COI sequences for a priori labeled groups. Values of this statistic lie on a continuum, with values of 0 indicating a random geographic distribution of sequences, and values of 1 indicating complete exclusive ancestry. We used collecting localities as a priori grouping variables; exclusive ancestry of COI sequences collected from a focal location implies limited (or non-existent) female-based gene flow among sampled locations. All analyses were conducted using the *gsi* website (http://www.genealogicalsorting.org/), with statistical significance assessed using 10,000 permutations of group labels on a fixed tree topology. The ML tree resulting from a no partitions RAxML analysis of an "all haplotypes" matrix (i.e., duplicate haplotypes not collapsed) was used as an input tree.

Yule-coalescent species delimitation.—The generalized mixed Yule-coalescent (GMYC) model (Pons et al. 2006; Monaghan et al. 2009) was used to identify genealogical clusters that may also correspond to cryptic species lineages. This model relies upon an expected difference in branching time intervals

Table 2.—PCR primer information. Primer references as follows: C1-J-1751SPID, C1-N-2568, C1-N-2776 (Hedin & Maddison 2001); C1-J-1718 (Simon et al. 1994); C1-J-1598HYPO, C1-J-1751MG, C1-J-1751SHE, C1-J-1751CO, C1-N-2568TH (this study). Primers marked with an asterisk were used in sequencing reactions.

PCR Primers	Taxon
*C1-J-1598HYPO, 5′-CGRGTWGAGTTRGGGCAAGT-3′	*H. pococki, H. sheari, H. coylei*
*C1-J-1718, 5′-GGAGGATTTGGAAATTGATTAGTTCC-3′	*H. pococki, H. sheari, H. coylei*
*C1-J-1751SPID, 5′-GAGCTCCTGATATAGCTTTTCC-3′	*H. thorelli*
*C1-J-1751MG, 5′-GGAGCTCCCGATATGGCGTTCCC-3′	*H. pococki, H. sheari, H. coylei*
*C1-J-1751CO, 5′-GGAGCGCCGGATATAGCGTTTCC-3′	*H. pococki, H. sheari*
C1-J-1751SHE, 5′-GGAGCACCAGAYATAGCATTTCC-3′	*H. pococki, H. sheari*
*C1-N-2568, 5′-GCTACAACATAATAAGTATCATG-3′	*H. pococki, H. sheari, H. coylei*
*C1-N-2568TH, 5′-GCCACAACGTAATAAGTATC-3′	*H. pococki, H. sheari*
*C1-N-2776, 5′-GGATAATCAGAATATCGTCGAGG-3′	*H. pococki, H. sheari*

between species (modeled as a stochastic birth-only Yule process) as compared to branching time intervals within species (modeled as a neutral coalescent process). Maximum likelihood is used to fit the GMYC model to an ultrametric tree to identify a threshold time (T) that corresponds to the Yule-coalescent transition (i.e., speciation). The model has been extended to allow multiple threshold times in a single phylogeny (see Monaghan et al. 2009) and has been used in many species delimitation studies in arthropods (e.g., Pons et al. 2006; Papadopoulou et al. 2008; Monaghan et al. 2009; Vuataz et al. 2011; Hamilton et al. 2011).

The three-partitions RAxML tree was used as input in GMYC analyses conducted using statistical packages implemented in R version 2.13.0. The *chronopl* function was used to transform the RAxML tree to an ultrametric tree using penalized likelihood (Sanderson 2002), and the *multi2di* function was used to randomly resolve polytomies in the ultrametric tree. Both functions are implemented in the APE library, version 2.5.3 for R (Paradis et al. 2004; Paradis 2006). Single and multiple-threshold GMYC models were optimized using the R script available within the SPLITS package (http://r-forge.r-project.org/projects/splits/) using default scaling parameters (interval = c(0,10)).

Morphological variation.—The pedipalps of adult male spiders were imaged and examined for a sample representing all Appalachian species, including all major phylogroups within species (see Results). Three primary palpal features were examined as follows: shape of the median apophysis in prolateral view, shape of the conductor tip in prolateral view, and shape of the palpal tarsus in retrolateral view (see Forster et al. 1987, Figs. 39, 41). The left palp was removed and immersed in filtered 70% EtOH, and secured using KY-Jelly. Digital images were captured using a Visionary Digital BK plus system (http://www.visionarydigital.com), including a Canon 40D digital camera, Infinity Optics Long Distance Microscope, P-51 camera controller and FX2 lighting system. Individual images were combined into a composite image using Helicon Focus V5.1 software (http://www.heliconsoft.com/heliconfocus.html), which was then edited using Adobe Photoshop CS3.

RESULTS

New COI sequences (~ 900 bp) were generated for 261 individuals from 85 localities. The number of sequences collected per sampling location ranged from one to five, with an average of about three sequences per location (Table 1). All sequences can be translated to amino acids with the standard Invertebrate mitochondrial genetic code, and lack insertion/deletion characters or stop codons. Representative sequences from all sample sites, including a population set, have been deposited to GenBank (accession numbers in Table 1). Geographic location information is also available as a Google Earth KMZ file available upon request from the corresponding author.

Phylogenetic and network analysis.—RAxML searches using alternative partitioning strategies result in very similar tree topologies, with minor differences restricted to relationships between closely related sequences within terminal clades. Tree topologies resulting from different RAxML analyses have been deposited at the Interactive Tree of Life page (Letunic and Bork 2006, 2011; http://itol.embl.de/shared/mhedin). Results from the three partitions analysis are shown here (Fig. 3) and discussed below; Fig. 3 also includes bootstrap values resulting from all three partitioning strategies.

Mitochondrial gene trees support the monophyly (likelihood bootstrap > 80) of the southern Appalachian fauna, and support the monophyly of haplotypes sampled for *H. sheari, H. coylei, H. thorelli* and *H. gertschi* (Fig. 3). Monophyly is not recovered for *H. pococki*. Instead, COI sequences from this species are fragmented into five primary, geographically cohesive clades – named the "Virginia", "Elk", "Northeast", "Western" and "Central" clades (see Figs. 2, 3). Of these genetic clades, the "Virginia", "Northeast" and "Western" clades are supported (likelihood bootstrap > 80). Except for a well-supported *H. thorelli* plus *H. coylei* sister pairing, interspecific and inter-clade relationships are not supported (bootstrap < 80) in any analysis. Average K2P-corrected (Kimura 1980) pairwise genetic divergences among species and primary geographic clades are quite high, ranging from 10.6 to 15.8% (Table 3).

At shallower levels (e.g., within species and the primary geographic clades of *H. pococki*) there is considerable evidence for fractal genetic structuring. Sequence divergence among sites within species/primary clades is high, ranging from 1.9 to 14.6% (Table 3). As a point of comparison, Robinson et al. (2009) analyzed data for a taxonomically broad sample of congeneric spider species, and reported a mean K2P COI divergence between nearest interspecific neighbors (~ sister taxa) of 6.8%. Most divergences within species and geographic clades of *Hypochilus* exceed average interspecific divergence values seen in other spiders. This deep divergence within species and primary clades is geographically structured, with many well-supported, geographically cohesive nested clades

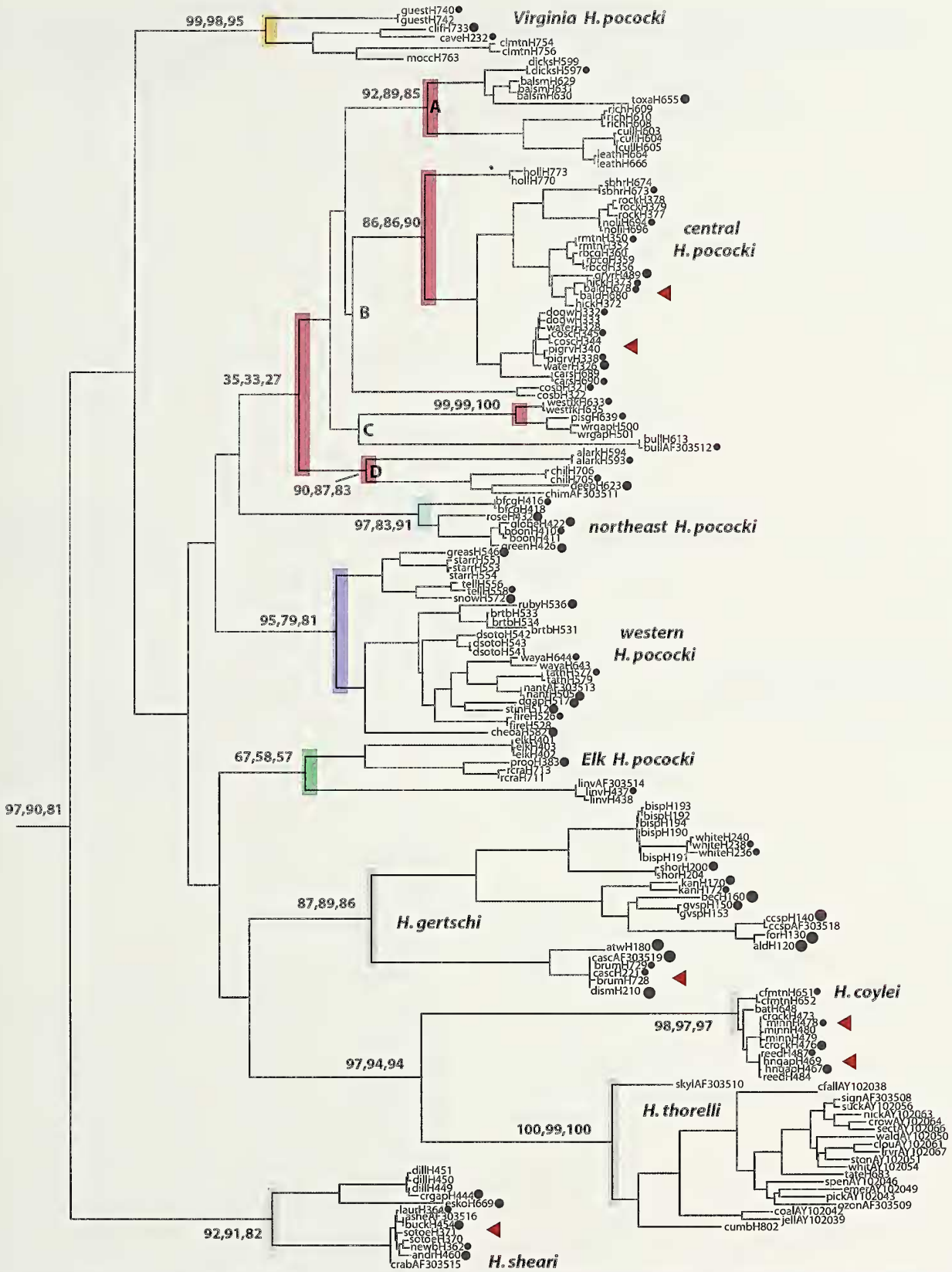

Figure 3.—ML tree reconstructed from three partitions analysis. Site acronyms are found in Table 1. Geographic clade colors for *H. pococki* correspond to those in Fig. 2. Bootstrap values resulting from no, two and three partitions analysis (respectively) shown for primary clades discussed in text. Cases of collection site non-exclusivity highlighted with red triangles. Gray circles associated with haplotype names indicate haplotypes shared by multiple specimens, with the smallest circles corresponding to $n = 2$, largest circles corresponding to $n = 4$ specimens. Node labels A–D in the "Central" *H. pococki* clade designate the four separate GMYC clusters resolved by the single threshold model.

Table 3.—Average K2P-corrected (Kimura 1980) mtDNA pairwise divergences within and between species and primary genetic clades (for *H. pococki*). A single, randomly chosen haplotype per sampled site was used; distances were computed in PAUP* version 4.0b10 (Swofford 2002).

	1	2	3	4	5	6	7	8	9	10
1. *H. thorelli*	0.0778	0.1404	0.1341	0.1137	0.1576	0.1508	0.1401	0.1132	0.1071	0.1216
2. *H. gertschi*	–	0.1150	0.137	0.138	0.1511	0.1469	0.1386	0.1269	0.1365	0.1408
3. *H. sheari*	–	–	0.0568	0.128	0.1492	0.1420	0.1271	0.1065	0.1118	0.1168
4. *H. coylei*	–	–	–	0.0190	0.1505	0.1438	0.1285	0.1151	0.1134	0.1357
5. *H. pococki*	–	–	–	–	0.1443	–	–	–	–	–
6. *H. pococki* (Central)	–	–	–	–	–	0.130	0.1367	0.1308	0.1360	0.1379
7. *H. pococki* (West)	–	–	–	–	–	–	0.094	0.1070	0.1149	0.1194
8. *H. pococki* (NE)	–	–	–	–	–	–	–	0.042	0.1157	0.1166
9. *H. pococki* (Elk)	–	–	–	–	–	–	–	–	0.146	0.1455
10. *H. pococki* (VA)	–	–	–	–	–	–	–	–	–	0.1036

(see Figs. 1–3). For example, samples of *H. sheari* are consistently separated into western and eastern subclades, samples of *H. gertschi* form three geographic subclades, samples of "Central" *H. pococki* fall into four subclades, etc.

Finally, this "clades within clades within clades" phylogenetic structuring extends to the level of local populations, where a pattern of location-specific genealogical exclusivity prevails (i.e., haplotypes from a sampling location form clades exclusive of other sampling locations). In total, we sampled two or more individuals from 81 locations, and recovered phylogenetic patterns indicative of haplotype mixing among locations in only six places on the ML tree (see Fig. 3). Of these six instances, TCS network analyses conclusively reveal haplotype sharing in only four cases, for the species *H. coylei*, *H. sheari*, and *H. gertschi* (Fig. 4).

Significant new distributional records.—Phylogenetic analyses confirm several new noteworthy distributional records for Appalachian *Hypochilus* taxa. This includes new northwestern records for *H. sheari* (esko, crgap, dill, Fig. 2; compare to Huff & Coyle 1992, fig. 12). Other significant records (compare to Forster et al. 1987, fig. 37) include the southernmost known record and a new county record for *H. gertschi* (brum, Washington County, Virginia, Fig. 1), the northeastern-most known record for *H. thorelli* (cumb, Lee County, Virginia, Fig. 1), a new county record for *H. pococki* in eastern Tennessee (clmtn, Hancock County, Tennessee, Fig. 2), and new western records for *H. pococki* in southeastern Tennessee (greas, starr, Polk County, Tennessee, Fig. 2).

Genealogical sorting index.—The 81 locations for which we sampled two or more sequences were defined as a priori labeled groups in *gsi* analyses. The average *gsi* value across all sites and species/genetic clades is relatively high (*gsi* = 0.917), with samples from only 14 locations exhibiting a *gsi* value less than 1 (Table 4). All *gsi* values are statistically significant under permutation ($P < 0.05$).

Yule-coalescent species delimitation.—A multiple thresholds model results in 54 Appalachian GMYC multiple-sequence clusters, whereas the single threshold model results in 11 Appalachian clusters. Because we view the multiple thresholds model as unrealistic (see Discussion), we prefer the single threshold model results. The eleven clusters defined by this analysis include *H. sheari*, *H. gertschi*, and the "Virginia", "Elk", "Northeast", and "Western" *H. pococki* genetic clades. The "Central" *H. pococki* clade is resolved as four separate GMYC clusters, corresponding to nodes labeled A–D on Fig. 3. The GMYC analysis collapses *H. coylei* and *H. thorelli* together into a single cluster. Although these latter two deseribed species share some male palpal features in common (e.g., shape of male conductor tip, see Catley 1994, Figs. 28, 29), they differ consistently in female spermathecal organ shape (Catley 1994, Figs. 14, 18) and have highly disjunct geographic distributions (Fig. 1).

Morphological variation.—All digital images have been deposited at Morphbank (www.morphbank.net). We imaged a single male spider from each of five different sampling locations (see Table 1) for the species *H. sheari* (Morphbank Nos. 691466–691475), *H. coylei* (Morphbank Nos. 691476–691485), *H. thorelli* (Morphbank Nos. 691496–691505) and *H. gertschi* (Morphbank Nos. 691486–691495). Examined features of male palps conformed to respective species deseriptions (Forster et al. 1987; Huff & Coyle 1992; Catley 1994), and we noted very little geographic variation within these described taxa. For *H. pococki* we examined a single male spider from 4–5 different sampling locations ($n = 22$, see Table 1) representing all primary geographic clades ("Virginia", "Elk", "Northeast", "Western" and "Central"; Morphbank Nos. 691421–691465). This sample included single males from each of the "Central" GMYC clusters. Although minor individual-level variation is evident, specimens from different primary *H. pococki* geographic clades are conserved in male palpal morphology (see www.morphbank.net, Fig. 5).

DISCUSSION

Population structure and phylogeography.—Hedin and Wood (2002) conducted in-depth population genetic analyses of *H. thorelli* based on a sampling of mitochondrial COI sequences for 85 individuals from 19 geographic sites. In this species there exists a pervasive pattern of low within-site versus high among-site mitochondrial genetic variation; i.e., most genetic variation is apportioned among, rather than within, sampled locations. Also, these authors found no COI haplotypes shared among sampling sites, despite the close geographic proximity (e.g., within 5 km) of certain sites. Based on these genetic patterns, Hedin and Wood (2002) argued for a 'fragmentation model' of extremely limited female-based gene flow, but recognized that geographic sampling at finer spatial scales eould possibly result in patterns consistent with genetic isolation by distance.

Our emphasis here was on general comparisons among taxa, not on distinguishing alternative models within a single taxon. These general comparisons reveal that mitochondrial population genetic structuring is similar among Appalachian

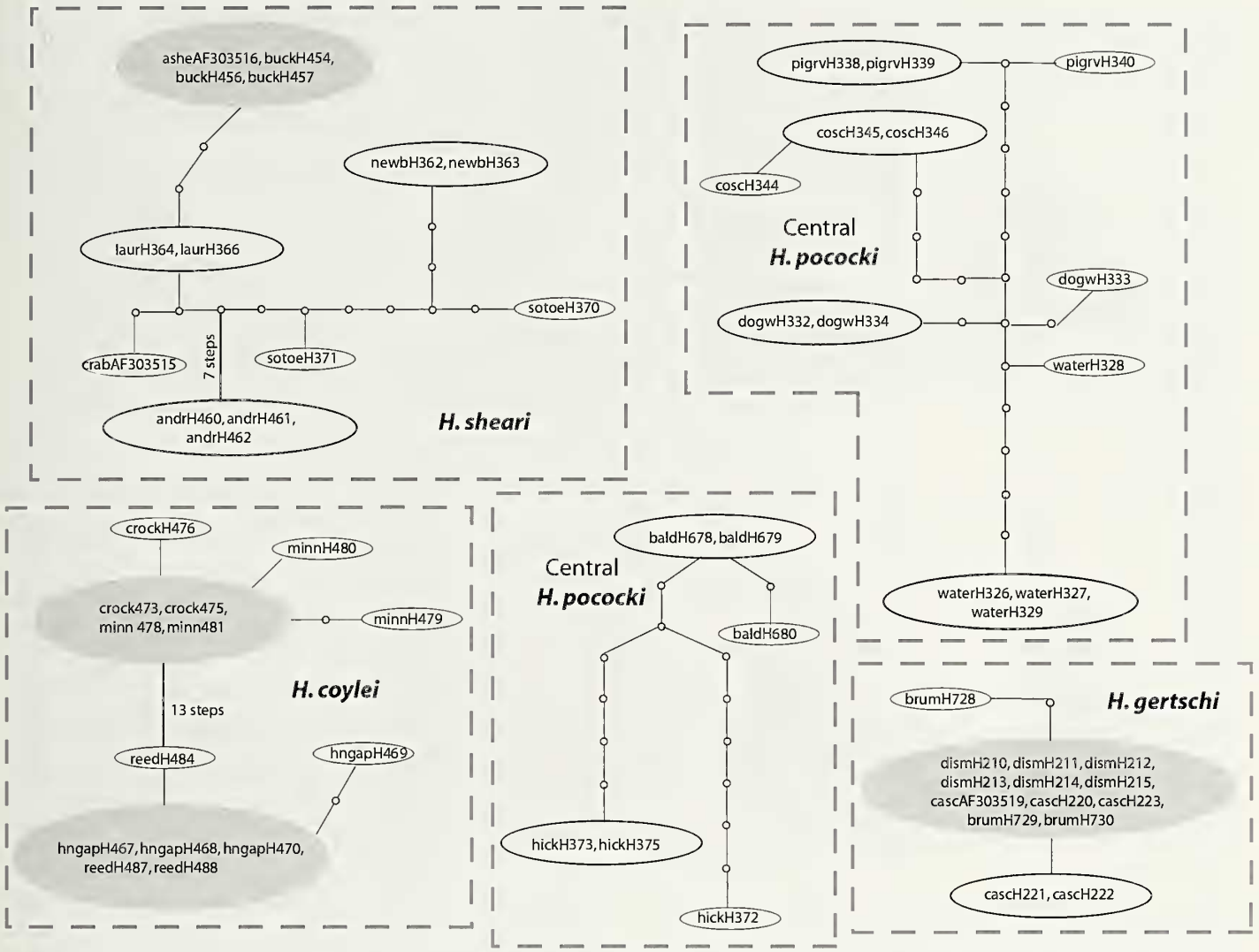

Figure 4.—TCS haplotype networks recovered at the 95% confidence level (Clement et al. 2000). Site acronyms correspond to those in Table 1.

Hypochilus species. This similarity exists despite the fact that these species are not expected to be biologically identical, and despite the fact that these species occur in different physiographic provinces of the southern Appalachians (i.e., southern Blue Ridge versus Cumberland Escarpment, etc., see Fig. 1), where we might expect rock outcrop availability and continuity to differ. For locations where we have sampled multiple specimens we find very little (if any) genetic variation, measured as the observed maximum number of nucleotide site differences per location (see Table 1). With few exceptions (see below), haplotypes from any single location form monophyletic "microclades", an inference supported by standard gene tree, network, and *gsi* analyses. Sequences in different microclades are obviously divergent, with divergence levels within phylogroups and species that are among the highest ever measured in spiders (Table 3). Overall, these patterns of mitochondrial structuring in southern Appalachian *Hypochilus* are consistent with a limited female-based gene flow scenario. This agrees with the lack of evidence for juvenile ballooning in these spiders, and with observations suggesting that the majority of adult dispersal is male-based (see Shear 1969; Fergusson 1972; Huff & Coyle 1992). This population subdivision is also consistent with many barriers to dispersal evident in the southern Appalachian Mountains.

We found a handful of instances consistent with either ongoing or historical gene flow. In both *H. coylei* and *H. sheari*, network analyses reveal identical haplotypes that are shared among sample sites (e.g., ashe & buck, crock & minn, hngap & reed – Fig. 4). Most of these cases involve locations that are relatively close in space (Fig. 2). Possible indirect evidence for gene flow is apparent for some sample locations that display high internal sequence divergence (see Table 1). For example, in "Western" *H. pococki*, haplotypes at *waya*, *brtb*, and *dsoto* are divergent (maximum divergences of 14, 19, and 16, respectively), even though these haplotypes form site-specific clades (Fig. 3). This pattern likely indicates gene flow from adjacent, but unsampled, demes. As argued in Hedin and Wood (2002), as the spatial scale of sampling more closely approximates individual dispersal distances, the pattern of zero gene flow breaks down, and the dynamic becomes more consistent with isolation by distance. The most obvious example of possible long-distance dispersal is seen in *H.*

Table 4.—GSI values.

Species	Site acronym	*gsi*	P value
H. pococki "central"	water	0.422	0.0001
"	dogw	0.664	0.0001
"	hick	0.664	0.0002
"	25 others	1	less than 0.002
H. pococki "Virginia"	4 sites	1	less than 0.002
H. pococki "Northeast"	boon	0.206	0.001
"	4 others	1	0.0001
H. pococki "western"	14 sites	1	0.0001
H. pococki "Elk"	4 sites	1	less than 0.002
H. sheari	sotoe	0.331	0.0009
"	dill	0.664	0.0001
"	6 others	1	less than 0.002
H. coylei	hngap	0.747	0.0001
"	crock	0.396	0.0003
"	minn	0.747	0.0001
"	reed	0.496	0.0001
"	cfmtn	1	0.0001
H. gertschi	brum	0.148	0.021
"	casc	0.491	0.0001
"	dism	0.491	0.0001
"	ald	0.797	0.0001
"	9 others	1	0.0001

gertschi, where identical haplotypes are shared among locations separated by large geographic distances (dism, casc, brum; Figs. 1, 4). Because northern populations of *H. gertschi* are genetically variable (Fig. 3), this may indicate population expansion toward the south from northern refugia.

Individual spiders and local populations of Appalachian *Hypochilus* species are almost always restricted to sheltered rock outcrop habitats (Hoffman 1963; Fergusson 1972; Forster et al. 1987; Huff & Coyle 1992; this study). As such, dispersal barriers must somehow coincide with areas where such habitat is lacking, although there are also instances where spiders are apparently lacking from seemingly suitable rocky habitat (e.g., see Huff & Coyle 1992, fig. 12), likely because of unsuitability of more general environmental factors (e.g., elevation, temperature, humidity, etc.). We suggest that future studies combine much denser geographic sampling with formal ecological niche modeling to understand how landscape factors impact the distribution of genetic variation in these spiders (i.e., landscape genetics, see Storfer et al. 2010).

Species delimitation in appalachian *Hypochilus*.—*Hypochilus* spiders possess a suite of shared biological characteristics consistent with what we term the "cryophilic syndrome". Commonalities of this syndrome include a restriction to specialized microhabitats that are naturally spatially fragmented (e.g., sheltered rock outcrops in mesic situations, etc.). Limited dispersal abilities, in combination with habitat specialization, result in pervasive population genetic subdivision and the evolution of divergent genetic groupings. Over longer evolutionary timescales, limited dispersal abilities result in many species that are geographically confined to small areas (short-range endemic taxa, sensu Harvey 2002; e.g., *H. coylei* and *H. gertschi*). In arrays of parapatric short-range endemic taxa, species syntopy is rare, probably because of ecological niche conservatism that prevents resource partitioning; this ecological niche conservatism likely plays an important role in speciation (following model of Wiens 2004). Finally, "cryophilic syndrome" taxa are also often morphologically conserved, perhaps reflecting stabilizing selection on morphology because of ecological niche conservatism. The combination of extreme population genetic subdivision with functional (i.e., ecological and morphological) conservatism implies that divergent genetic groupings often lack obvious functional divergence, or show only subtle functional divergence.

We are most familiar with taxa exhibiting the "cryophilic syndrome" in arachnids and other arthropods, although some vertebrate taxa also share these features (e.g., *Batrachoceps* salamanders, Joekusch & Wake 2002; Wake 2006; *Xantusia* night lizards, Sinclair et al. 2004; Leavitt et al. 2007). In arachnids, integrative studies assessing both genetic and functional divergence have revealed patterns consistent with this syndrome in many small-bodied harvestmen taxa (e.g., Boyer et al. 2007; Thomas & Hedin 2008; Hedin & Thomas 2010; Schönhofer & Martens 2010). Ground-dwelling mygalomorph spiders are also conspicuous in this regard (Bond et al. 2001; Hendrixson & Bond 2005; Arnedo & Ferrandez 2007; Starrett & Hedin 2007; Bond & Stockman 2008).

When divergent genetic groupings lack obvious functional divergence, the process of species delimitation is very challenging, and must incorporate multiple lines of evidence. This is indeed the case for southern Appalachian *Hypochilus*. The interpretation of contrasting data patterns is difficult, with genetic data suggesting high divergence and many separate lineages, whereas functional data suggest limited divergence and fewer distinct lineages. This contrast provides interesting insight into how these lineages evolve, but what are the species limits? A "many cryptic species" hypothesis would include as distinct species four named *Hypochilus* taxa (*H. sheari, H. coylei, H. thorelli, H. gertschi*), plus divergent phylogroups within *H. pococki*. Under the GMYC single threshold model, four additional species would be resolved within "Central" *H. pococki*. It is important to note that all of these genetic groups possess qualities consistent with species status under many different species criteria (see Sites & Marshall 2004), including reciprocal monophyly, high interspecific divergence, and contiguous geographic distributions (Figs. 2, 3). Also, a geographic pattern of several species with relatively small and allopatric distributions is expected for organisms with low vagility, particularly in a region as topographically complex as the southern Appalachians.

However, there are several problems with this "many cryptic species" interpretation. First, because mtDNA reflects only maternal genetic histories, it is not known whether observed population genetic structuring extends to both genomes. Is male-based gene flow in these spiders extensive enough to act as a cohesive evolutionary force? Second, theory demonstrates that deep mitochondrial genealogical breaks can arise stochastically in low dispersal systems (Irwin 2002; Kuo & Avise 2005), again making it difficult to interpret the significance of observed genetic patterns. Finally, even if the genetic system used here was biparental, fractal genetic structuring makes it difficult to define boundaries of higher-

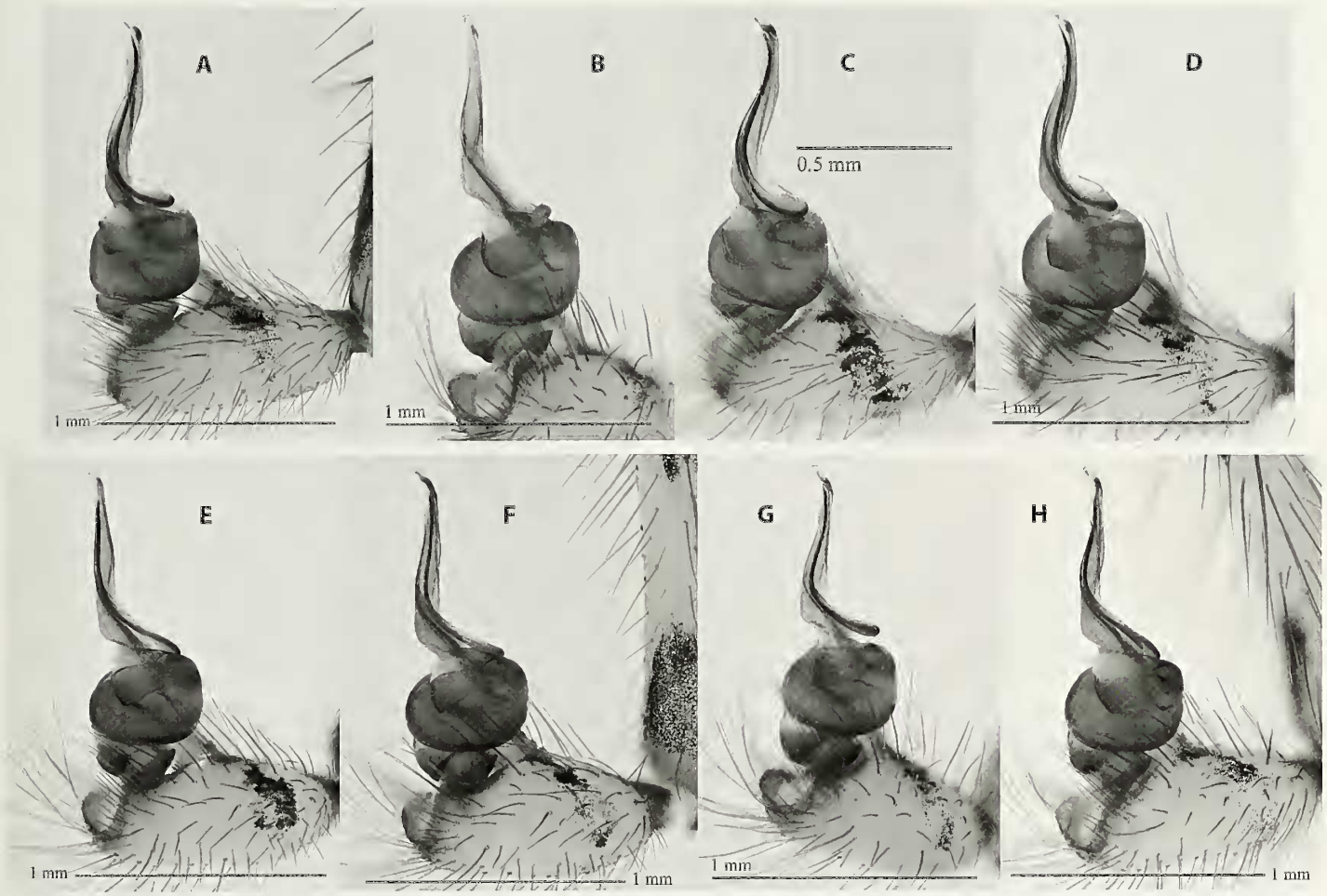

Figure 5.—Representative variation in male palpal morphology in *H. pococki*: A) "Central" Clade, GMYC cluster A, Toxaway; B) "Central" Clade, GMYC cluster B, Dogwood Flats; C) "Central" Clade, GMYC cluster C, West Fork Pigeon River; D) "Central" Clade, GMYC cluster D, Chilhowee; E) "Elk" Clade, Elk River; F) "Northeast" Clade, Green Mtn.; G) "Virginia" Clade, Cliff Mtn.; H) "Western" Clade, Greasy Creek. All views prolateral.

level units, e.g., phylogeographic units versus species, because genealogical breaks are ubiquitous. Some authors have argued that significant intraspecific population structure may confound GMYC analyses (Lohse 2009; but see Papadopoulou et al. 2009), and we reject the multiple thresholds GMYC model (implying 54 species) for this reason.

In light of the potential limitations of mitochondrial gene tree data discussed above, we favor a more conservative perspective (based on male genitalic morphology in particular), and do not recommend taxonomic changes at this time. This conservative, functional divergence perspective treats different named species as distinct, as these taxa differ in genital morphology. This interpretation is not without difficulties. First, we must accept the genetic non-monophyly of a species-level taxon (i.e., *H. pococki*), although it could be argued that this non-monophyly reflects inaccurate gene tree estimation (e.g., due to mutational saturation, etc.). Second, if we accept the premise that separate species can be morphologically cryptic (at least as considered with current technology; see Saez & Lozano 2005; Bickford et al. 2007; Daniels et al. 2009), then it is clearly possible that a conservative perspective potentially undersplits Appalachian *Hypochilus* species diversity. To further test species delimitation hypotheses in this challenging group we recommend a multigenic genealogical approach. This would include the collection of DNA sequence data from many independent nuclear markers, clearly feasible given the increase in genomics tools (e.g., via next-generation sequencing) for non-model systems (e.g., see Thomson et al. 2010). Such data could then be combined with new methods for species delineation (Yang & Rannala 2010; Leaché & Fujita 2010) to delimit species as groups that represent genetic clades recovered for multiple loci, with or without functional diagnosability. The research presented here pinpoints geographic regions and potential cryptic lineages to target under such a study plan.

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